The preparation and characterization of locust vitellogenin messenger RNA and the synthesis of its complementary DNA

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Poly(A)⁺ (polyadenylated) RNA was isolated from vitellogenic female-locust fat-body by LiCl/urea extraction and poly(U)–Sephrose 4B affinity chromatography. Agarose-gel electrophoresis of this poly(A)⁺ RNA under denaturing conditions shows the presence of a high-molecular-weight species (>31S, 7100 nucleotides) as the major species, which is absent from the RNA prepared from male-locust fat-body. Inclusion of this poly(A)⁺ RNA in a mRNA-dependent reticulocyte-lysate system directs the synthesis of polypeptides that could be immunoprecipitated with monospecific antibodies against locust egg vitellin. DNA complementary (cDNA) to the poly(A)⁺ RNA was synthesized, and back-hybridization of the cDNA to its template reveals a major abundant species comprising about 45% of the total poly(A)⁺ RNA hybridizing with a $R_0t_{1/2}$ of 2×10^{-2} mol·litre⁻¹·s. Abundant cDNA isolated from the total cDNA hybridizes to poly(A)⁺ RNA with a $R_0t_{1/2}$ of 9×10^{-3} mol·litre⁻¹·s. There are 9.1×10^3 copies of vitellogenin mRNA per cell of vitellogenic female-locust fat-body, comprising 55% of the poly(A)⁺ RNA and equivalent to 0.7% of total cellular RNA.

Locust vitellogenesis exhibits several unusual characteristics, differing in principle or in degree from avian or amphibian vitellogenesis. The proteolytic cleavage of vitellogenin to lipovitellin and phosvitin occurs in egg-laying vertebrates during its uptake into the oocyte (Bergink & Wallace, 1974), whereas the major proteolytic processing of locust vitellogenin already occurs in the fat-body, where it is synthesized before it is secreted into the haemolymph (Chen *et al.*, 1978). There is no analogy to phosvitin in insect vitellogenin, and only few of the available serine residues are phosphorylated (Engelmann & Friedel, 1974; M. Pines & S. W. Applebaum, unpublished work). The induction of vitellogenin-mRNA synthesis in vertebrates is controlled by oestrogens, and their injection into male chicken

or *Xenopus* induces the transcription of vitellogenin mRNA in the liver and subsequent vitellogenin synthesis and secretion (Gruber *et al.*, 1976; Tata & Smith, 1979). In most insects studied, juvenile hormone, which is present in males as well as in females, controls vitellogenesis. In most species, vitellogenesis has been difficult to induce in male insects, although its induction in male cockroaches has been demonstrated (Mundall *et al.*, 1979), and minute quantities of vitellogenin had also been detected earlier in male silkworm (*Hyalophora cecropia*) (Telfer, 1954) and in the large milkweed bug (*Oncopeltus fasciatus*) (Kelly & Telfer, 1977). Besides the inherent evolutionary significance in determining whether these differences in processing of vertebrate and invertebrate vitellogenins are reflected in the characteristics of the vitellogenin gene itself, which is regarded as highly conserved in vertebrates, quantitative information about the relative abundance of this gene in insects and the extent of its homology with vertebrate vitellogenin genes may offer an insight into the hormonal regulation of expression of this gene. As a prerequisite for such analyses, it is essential to isolate and characterize vitellogenin mRNA and synthesize the corresponding cDNA probes for molecular hybridization. In this present study, we report the preparation of a

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Abbreviations used: cDNA, complementary DNA; poly(A)⁺ RNA, polyadenylated RNA; SDS, sodium dodecyl sulphate; C_0t , product of DNA concentration and the time of incubation (expressed as mol of deoxyribonucleotides·litre⁻¹·s); R_0t , product of RNA concentration and the time of incubation (expressed as mol of ribonucleotides·litre⁻¹·s); $R_0t_{1/2}$, R_0t value which is needed to allow hybridization to proceed halfway to completion.

partially purified high-molecular-weight poly(A)⁺ RNA from female-locust fat-body, which directs the synthesis of locust vitellogenin in a reticulocyte lysate cell-free system. This RNA was reverse-transcribed to give a high-molecular-weight cDNA preparation, from which the most abundant fraction was isolated by back-hybridization to its template. The quantification of vitellogenin mRNA in vitellogenic female-locust fat-body with the use of such a probe is also described.

Materials and methods

Materials

Vitellogenic female locusts were a gift from The Centre for Overseas Pest Research, London W.8, U.K. [³H]dCTP (sp. radioactivity 21 Ci/mmol), [³H]TTP (sp. radioactivity 78 Ci/mmol), [³²P]dCTP (sp. radioactivity 3000 Ci/mmol) and [³⁵S]methionine (sp. radioactivity 700 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Deoxyribonucleoside triphosphates, actinomycin D, S₁ nuclease and creatine kinase were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K. (dT)₁₄ and proteinase K were obtained from Boehringer-Mannheim, London W.5, U.K. Poly(U)-Sephacrose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Micrococcal nuclease was obtained from P-L Biochemicals, Milwaukee, WI, U.S.A. Avian-myeloblastosis-virus reverse transcriptase was a gift from Dr. J. W. Beard (Life Sciences Inc., St. Petersburg, FL, U.S.A.). All other chemicals were of analytical grade and were obtained from Sigma, London, or BDH Chemical Co., Poole, Dorset, U.K.

Extraction of total RNA from the fat-body

Fat-bodies were excised from vitellogenic females and immediately frozen in liquid nitrogen. Total RNA from 10–15 g batches of fat-bodies was extracted by the LiCl/urea procedure modified from Rhoads (1975) and Roskam & Rougeon (1979). Approx. 10–15 g of fat-bodies were homogenized at 4°C, with a motor-driven Potter-Elvehjem homogenizer, in 100–150 ml of 3 M-LiCl/6 M-urea containing 100 mM-sodium acetate, pH 5.5, heparin (200 µg/ml) and 0.1% SDS (medium A). The homogenate was then diluted to 300 ml with medium A. After 10 h on ice, the homogenate was centrifuged at 16000 rev./min at 4°C for 45 min in a Beckman J21C instrument in a JA21 rotor. The lipid layer was removed as a plug from the top of the tubes and the supernatant discarded. The pellet was washed twice with 100 mM-sodium acetate, pH 5.5, containing 4 M-LiCl, 8 M-urea and 1% SDS. The final pellet was suspended in 2 ml of 1 M-sodium acetate buffer, pH 5.5, containing 1% SDS (medium B), and extracted with an equal volume of phenol saturated

in medium B without SDS. The aqueous phase together with the interphase was collected. The phenol phase was then re-extracted with medium B and the aqueous phase pooled with the first one. The combined aqueous phases were re-extracted with an equal volume of phenol saturated with medium B, followed by phenol/chloroform (1:1, v/v) and then finally with chloroform alone. The final aqueous phase was precipitated overnight at –20°C with 2.5 vol. of redistilled 96% (v/v) ethanol. The precipitate containing total RNA was recovered and washed twice with 96% ethanol, vacuum dried, dissolved in a small volume of sterile distilled water and stored at –70°C until use.

Isolation of poly(A)⁺ RNA

Poly(A)⁺ RNA was isolated from the total RNA by using poly(U)-Sephacrose 4B chromatography as described by Vassart *et al.* (1973). Approx. 100–200 A₂₆₀ units of total RNA in 2.5 ml of sterile water were heated at 90°C for 1 min, or at 70°C for 15 min, and diluted into 10 vol. of binding buffer [10 mM-Tris (pH 7.6)/300 mM-NaCl/1 mM-EDTA/0.2% SDS]. The diluted RNA (10 A₂₆₀ units/ml) was applied to a jacketed poly(U)-Sephacrose column (2 cm × 10 cm; bed volume 2 ml), which was equilibrated in binding buffer at room temperature. The flow rate was kept at 30 ml/h. The flow-through fraction was twice re-applied and the column was then washed with 50 ml of binding buffer to remove all unbound RNA. When no more RNA was eluted from the column (based on the A₂₆₀), the temperature of the column was raised to 50°C and 3 ml of elution buffer [10 mM-Tris (pH 7.6)/1 mM-EDTA/0.2% SDS] kept at 50°C was added on to the column, in six 0.5 ml batches. The eluted material was made to 0.3 M-sodium acetate, pH 5.5, and precipitated overnight at –20°C with 2.5 vol. of redistilled 96% ethanol. The precipitate was recovered and processed as described for total RNA.

Isolation of high-molecular-weight DNA

High-molecular-weight DNA was isolated from locust fat-body as described by Gross-Bellard *et al.* (1973).

Analytical agarose-gel electrophoresis

RNA and cDNA preparations were electrophoresed in 1% agarose slab gels under denaturing conditions (McMaster & Carmichael, 1977). Globin mRNA, rat liver and *Escherichia coli* rRNA species were used as markers. Electrophoresis was carried out at room temperature at 100 V for 4 h. All gels were stained with ethidium bromide (1 µg/ml) and photographed under u.v. light by using an orange filter and Polaroid type 55 film. Gels containing cDNA preparations were radioautographed after drying them on to a Whatman 3MM filter paper, or

processed for fluorography as described by Laskey *et al.* (1977).

Synthesis of cDNA

DNA complementary to total poly(A)⁺ RNA from female locust fat-bodies was synthesized by the method of Williams & Penman (1975) with minor modifications (Smith *et al.*, 1979). The yield (μg of cDNA/ μg of input RNA) was approx. 1.2%.

RNA-excess hybridization

In general, cDNA–RNA-excess hybridization reactions were carried out in 2 μl incubation mixtures sealed in silicone-treated 5 μl graduated capillaries (Clay–Adams) as described by Deeley *et al.* (1977), with minor modifications. RNA was present in 10^3 – 10^4 -fold excess relative to the amount of labelled cDNA used. The hybridization buffer used was 0.01 M-Hepes [4-(2-hydroxymethyl)-1-piperazine-ethanesulphonic acid], pH 7.0, containing 0.6 M-NaCl and 0.002 M- Na_2EDTA . Incubations were carried out at 69°C for various times up to a maximum of 24 h and the reaction was stopped by immersing the capillaries in an ice/ethanol bath. The extent of hybridization was determined by digestion with S_1 nuclease (Milcarek *et al.*, 1974; James & Tata, 1980).

DNA-excess hybridizations

High-molecular-weight DNA was randomly sheared to fragments of 450 ± 100 nucleotides in length by shearing in a VirTis disintegrator in 2 vol. of glycerol at -70°C (Britten *et al.*, 1974). The sheared DNA was electrophoresed on 1% agarose gel, as described above, to determine the size range of the fragments. DNA–cDNA hybridizations were performed in vast DNA excess, usually 10^6 -fold excess (10 – 26 mg/ml) over cDNA, to saturate complementary sequences. cDNA and sheared DNA were taken up in 50 mM-Tris/HCl (pH 7.0)/0.3 M-NaCl/1 mM-EDTA/0.1% SDS, and incubated in 3–5 μl volumes in silicone-treated capillaries. They were denatured for 10 min at 100°C and then allowed to hybridize at 69°C for various times extending up to 500 h. The hybridization reaction was stopped and processed as described above.

Thermal-denaturation profiles

Purified cDNA against locust vitellogenin mRNA was hybridized against locust genomic DNA in 10^6 -fold DNA excess in 50 mM-Tris/HCl (pH 7.0)/0.3 M-NaCl/1 mM-EDTA/0.1% SDS to an equivalent $C_{\theta t}$ value of 10^6 . Samples of the hybrids were then incubated in the same buffer for 10 min at different temperatures in order to determine the ‘melting’ temperature of the hybrids. The reaction mixture was quickly frozen in liquid nitrogen, and the extent of double-strandedness at each tempera-

ture was assayed by using single-strand-specific S_1 nuclease as described above.

Isolation of cDNA complementary to abundant mRNA

cDNA corresponding to abundant species of mRNA was isolated from the total cDNA by a back-hybridization procedure by the method of Gordon *et al.* (1978). Approx. 50 ng of cDNA and 250 ng of poly(A)⁺ RNA in 20 μl of hybridization buffer were hybridized to a $R_{\theta t}$ value of $2 \times 10^{-2} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$. The hybrids were processed with S_1 nuclease, and ‘highly abundant’ cDNA was recovered as described by Gordon *et al.* (1978).

Cell-free translation and analysis of translation products

Total RNA or poly(A)⁺ RNA isolated from vitellogenic locust fat bodies as described above was translated in micrococcal-nuclease-treated reticulocyte-lysate cell-free system (vol. 12.5 μl) (Pelham & Jackson, 1976). After 90 min incubation at 30°C the samples were either directly processed for SDS/polyacrylamide-gel electrophoresis or immunoprecipitated with anti-vitellogenin antibodies whose specificity and purity were checked by Ouchterlony double-immunodiffusion analysis (Harry *et al.*, 1979). The labelled translation products, before and after immunoprecipitation, were electrophoresed on SDS/9%-polyacrylamide gels (Laemmli, 1970) and the gels processed for radioautography.

Results and discussion

RNA extraction from locust fat-body

Initial attempts to purify the mRNA coding for vitellogenin from vitellogenic locusts, by using SDS, chloroform, phenol, and various ribonuclease inhibitors, gave degraded vitellogenin mRNA in low yields. It was not until we resorted to an RNA-extraction procedure that virtually totally inhibited ribonuclease activity at the very beginning that vitellogenin mRNA was obtained that was both intact and in good yields. This method involved freezing the locust fat-bodies in liquid nitrogen and then homogenizing them in a strongly denaturing medium (6 M-urea), while the high salt content (3 M-LiCl) effectively dissolves DNA, low-molecular-weight RNA and glycogen, leaving high-molecular-weight RNA in the pellet after centrifugation (Tata & Baker, 1975). When the integrity and relative amount of RNA were examined by agarose-gel electrophoresis under denaturing conditions, as shown in Fig. 1(a), total RNA from vitellogenic female-locust fat-bodies extracted by this LiCl/urea method displayed a high-molecular-weight RNA, detected by ethidium bromide at the position corresponding to a size of 31 S (Fig. 1b). The larger

Fig. 1. Agarose-gel electrophoresis of glyoxal/dimethyl sulphoxide-denatured locust fat-body RNA

(a) Photograph of staining with ethidium bromide of electrophoretic gel showing the migration of RNA. For this experiment 5–20 μ g of RNA was denatured in 1M-glyoxal in 50% dimethyl sulphoxide before electrophoresis in 1% agarose gel (McMaster & Carmichael, 1977). Lanes 1 and 8, 10 μ g of rat rRNA (28S and 18S) as marker; lane 4, 20 μ g of *Escherichia coli* rRNA (23S and 16S); lanes 2 and 3, 10 μ g of total RNA isolated from fat-body of male and female locusts, respectively; lanes 5 and 6, 1 and 15 μ g, respectively, of poly(A)⁺ RNA isolated from female-locust fat-body; lane 7, 10 μ g of rabbit globin (9S) mRNA. Arrows denote the migration of vitellogenin mRNA at the 31S position. (b) Estimation of molecular weight of presumptive vitellogenin mRNA (▲) and an unidentified abundant RNA in the poly(A)⁺ RNA fraction (△).

rRNA component is nicked *in vivo* in a manner characteristic for insect rRNA (Applebaum *et al.*, 1966), so that under denaturing conditions of electrophoresis its mobility increases from that of an RNA of 27S to that of 20S. The total RNA extracted by the LiCl/urea procedure exhibited an A_{260}/A_{280} ratio of 1.85–1.95. The 31S RNA could not be observed in similar RNA preparations from male-locust fat-bodies. The yield of poly(A)⁺ RNA from vitellogenic female fat-bodies is 5 times that obtained from an equivalent amount of male fat-bodies, presumably reflecting the extensive proliferation of rough endoplasmic reticulum in vitellogenic females (Couble *et al.*, 1979).

Affinity chromatography of RNA

Chromatography of RNA isolated from fat-bodies of vitellogenic locusts on oligo(dT)-cellulose resulted in poor recovery of vitellogenin mRNA and considerable contamination of the poly(A)⁺ RNA fraction with rRNA (cellulose has a greater tendency to bind rRNA non-specifically than does Sepharose). On the other hand, poly(U)-Sepharose binds mRNA having relatively shorter poly(A) tails more efficiently than does oligo(dT)-cellulose (Shapiro & Schimke, 1975). With a standard procedure of binding of polyadenylated RNA to poly(U)-Sepharose at high ionic strength and thermal elution at 50°C with a low-ionic-strength buffer, a single-step enrichment of the 31S RNA, at high yield, was attained. Another RNA migrating as a 14S species was also recovered as a minor component, along with traces of rRNA and some very-high-molecular-weight RNA species. The 31S RNA obtained by affinity chromatography is unique to vitellogenic females. The cDNA obtained from this 31S poly(A)⁺ RNA appears to contain almost full-length transcripts (see below), whereas poly(A)⁺ RNA isolated by oligo(dT)-cellulose chromatography did give transcripts of smaller size.

Translation of poly(A)⁺ RNA

When the poly(A)⁺ RNA isolated on a poly(U)-Sepharose column was translated in a nuclease-treated reticulocyte-lysate cell-free system, it was found to be a less efficient template in promoting incorporation of [³⁵S]methionine into protein as compared with a pure globin mRNA assayed simultaneously (results not shown) as a control. The incorporation was a function of the concentration of poly(A)⁺ RNA. Analysis of the translation products on a SDS/9%-polyacrylamide gel (Fig. 2) showed a number of high-molecular-weight radioactive protein bands, including one which appeared as a high-molecular-weight translation product exceeding 250 000 daltons. In order to establish whether or not this high-molecular-weight protein was the primary translation precursor of processed vitello-

Fig. 2. SDS/polyacrylamide-gel electrophoresis of [^{35}S]methionine-labelled polypeptide products of translation of female-locust fat-body poly(A) $^{+}$ RNA in a rabbit reticulocyte cell-free translation system

Samples (25–125 ng) of poly(A) $^{+}$ RNA were transplanted in duplicate in 12.5 μl of micrococcal-nuclease-treated rabbit reticulocyte lysate and analysed by electrophoresis on SDS/9%-polyacrylamide gel as described by Laemmli (1970), followed by radioautography (Shore & Tata, 1977). Lanes 1–7, before immunoprecipitation; lanes 8–13, after immunoprecipitation with monovalent antibody to locust egg vitellin in the presence of non-radioactive vitellin (Harry *et al.*, 1979). Input RNA translated: lane 1, 25 ng; 2, 50 ng; 3, 75 ng; 4, 100 ng; 5, 125 ng. Lanes 6 and 7: endogenous [^{35}S]methionine incorporation in the absence of added locust RNA. Lanes 8–13 represent the immunoprecipitated samples of duplicates corresponding to the samples in lanes 1–6 respectively. Arrow indicates the position of the non-radioactive *Xenopus* vitellogenin marker electrophoresed separately.

genin, we performed immunoprecipitation of the products of translation, using purified monospecific antibody against locust egg vitellin in the presence of non-radioactive authentic vitellin added as carrier. The anti-vitellin antibody, which precipitates vitellogenin equally well (Gellissen *et al.*, 1976), also precipitated the high-molecular-weight translation product (Fig. 2). The intensity of this band of immunoprecipitated protein increased with increasing RNA concentration in the translation assay. No such immunoprecipitable radioactive material could be seen in the reticulocyte lysates incubated with rabbit globin mRNA. This demonstrates that the high-molecular-weight protein coded for by the poly(A) $^{+}$ RNA from female locust fat-bodies is indeed vitellogenin which has not yet been processed by limited proteolytic cleavage to its final products. Furthermore, other experiments (not described here) in which the poly(A) $^{+}$ RNA was fractionated first on a denaturing sucrose gradient and then each fraction translated separately (James & Tata, 1980) showed that the major mRNA was a 31 S species coding for vitellogenin. Apart from the 250000-dalton protein, there were two other bands corresponding to proteins of about 50000 daltons in size in the translation assay which were also immunoprecipitable by the antivitelin antibody.

Synthesis of DNA complementary to poly(A) $^{+}$ RNA

Since the above translation experiments suggested that the major fraction of the total poly(A) $^{+}$ RNA was most likely to be vitellogenin mRNA, we decided to use this preparation as a template for the synthesis with reverse transcriptase of cDNA to vitellogenin mRNA. For these studies it was preferable to maximize the length of the cDNA transcript, particularly because of our use of back-hybridization to a low R_{ot} value. As a test for the experimental conditions used, we were able to reverse-transcribe the globin mRNA into a full-length cDNA transcript of 600 nucleotides. The cDNA transcript of the poly(A) $^{+}$ RNA from vitellogenic female-locust fat-bodies is heterogeneous in size, corresponding to a range of 30S to 9S on electrophoresis in agarose gel (Fig. 3). The upper limit of this size range indicates that some full-length cDNA transcripts of vitellogenin mRNA were synthesized. The efficiency of transcription was 1.2% of input poly(A) $^{+}$ RNA, one-tenth that obtained with the globin mRNA control, which is reasonable if one takes into account the large size of vitellogenin mRNA. Some 2–5% of the cDNA was double-stranded, as judged by its resistance to digestion with S_1 nuclease both before and after denaturation at 100°C for 5 min in 200 mM-NaCl.

Fig. 3. Agarose-gel electrophoresis of ^{32}P -labelled cDNA to locust fat-body RNA

Radioautogram showing the size distribution of ^{32}P -labelled cDNA synthesized on total poly(A)⁺ RNA and cDNA enriched for vitellogenin-mRNA sequences by back-hybridization to low R_0t values. Lane 1, cDNA (800000 c.p.m.) to total poly(A)⁺ RNA; lanes 2 and 3, cDNA (300000 c.p.m. each) to abundant mRNA (vitellogenin mRNA) isolated as described by Gordon *et al.* (1978) with limited back-hybridization to a R_0t value of 4×10^{-2} and 6×10^{-2} , respectively. The samples were denatured in 1M-glyoxal in 50% dimethyl sulphoxide, and electrophoresis was performed as described by McMaster & Carmichael (1977), followed by radioautography (Shore & Tata, 1977). The cDNA species in lanes 1–3 exhibited 2, 5 and 7% S_1 -nuclease resistance respectively. Nucleotide (nt) lengths were calculated from rRNA species from *E. coli* and rat liver electrophoresed in parallel as standards.

RNA-excess hybridization analysis using cDNA

Calculations of mRNA abundance based solely on translation assays *in vitro* are subject to the variable efficiencies with which different mRNA molecules are translated. Despite the fact that vitellogenin mRNA is the major RNA species in the poly(A)⁺ RNA preparation (Fig. 1a), and appar-

ently full-length translation products immunoprecipitable with anti-vitellin are obtained in a cell-free system, these are by no means the only proteins produced. Quantitative translation assay for abundance of the vitellogenin mRNA is not feasible under these conditions. An alternative and more reliable method of determining the frequency with which a given mRNA sequence occurs in a population of mRNA molecules is by cDNA complexity analysis (Bishop *et al.* 1974). The complexity of poly(A)⁺ RNA can be estimated by hybridization with its cDNA under conditions of RNA excess and comparison of the observed reaction rate with that of a kinetic standard derived from a highly purified mRNA sequence of known sequence complexity (expressed as length in nucleotides). Under the reaction conditions used here, purified ovalbumin mRNA reacts with its cDNA with the $R_0t_{1/2}$ $1.3 \times 10^{-3} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$, with a sequence complexity of 1600 nucleotides. Fig. 4 shows the results obtained when cDNA transcribed from a poly(A)⁺ RNA from female vitellogenic locust fat-bodies is hybridized back to its template. A maximum value of reactivity of 80% was obtained from the cDNA probes. The curve drawn through the open circles shows the presence of at least two abundance classes of mRNA species, a major abundance class representing approx. 44% of the total poly(A)⁺ RNA species transcribed and which reacts with a $R_0t_{1/2}$ of $2 \times 10^{-2} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$, whereas the remaining 36% of the poly(A)⁺ RNA formed a less abundant,

Fig. 4. Kinetics of hybridization of poly(A)⁺ RNA isolated from female-locust fat-body to unfractionated [^{32}P]cDNA transcribed from it (O) and to purified vitellogenin [^{32}P]cDNA (●)

RNA-excess hybridization was performed as described in the text. Two major classes of RNA can be discerned. The abundant class (44%) of RNA hybridizes with an apparent $R_0t_{1/2}$ of $2 \times 10^{-2} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$, and a less-abundant class (36%) hybridizes at a $R_0t_{1/2}$ of $8 \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$. Purified vitellogenin cDNA hybridizes with a $R_0t_{1/2}$ of $9 \times 10^{-3} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$.

complex class which hybridizes more slowly at a $R_0t_{1/2}$ of $8 \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$. The actual $R_0t_{1/2}$ ($2 \times 10^{-2} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$) obtained for the highly abundant class can be corrected to a value of $9 \times 10^{-3} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$ because of the 80% reactivity of the components and fractional representation (44%) of the abundant component among the total poly(A)⁺ RNA (Gordon *et al.*, 1978). The apparent complexity of this class of mRNA is 12600 nucleotides, which is 1.8 times the value derived from the sequence complexity of the 31S RNA (2.3×10^6 daltons, or 7100 nucleotides) recovered as a major fraction in the poly(A)⁺ RNA from vitellogenic locust fat-bodies. The discrepancy may arise from the contamination with rRNA of the poly(A)⁺ RNA or that it reflects the presence of more than one species of locust vitellogenin mRNA, as has been suggested by Chen (1980).

Isolation of cDNA complementary to highly abundant mRNA, and its amount in female locust fat-body

An abundant class of cDNA species was isolated by preparative RNA-excess hybridization to a R_0t of $6 \times 10^{-2} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$. At this R_0t value we expected that only the highly abundant mRNA population would hybridize to its own cDNA. Hybridization was followed by S_1 -nuclease digestion of all sequences that had not reacted, proteinase-K digestion and phenol extraction to remove S_1 nuclease, and finally alkaline hydrolysis of the RNA sequences from the hybrid (Gordon *et al.*, 1978). When the 'abundant' cDNA was analysed by agarose-gel electrophoresis under denaturing conditions, a diffuse band corresponding to a size of 1.4×10^6 – 1.1×10^6 daltons or 4000–3000 nucleotides was observed. Similar attempts to synthesize full-length cDNA from vitellogenin mRNA species of *Xenopus* or chicken have not been successful (Wahli *et al.*, 1978; Ohno *et al.*, 1980). The average length of the cDNA transcribed was one-third the size of the vitellogenin mRNA, the interrupted transcription most probably signifying the presence within the vitellogenin mRNA of either an internal priming sequence or a stop signal for reverse transcriptase. The abundant locust cDNA (vitellogenin cDNA) transcribed in our experiments represents one-half of the theoretical full size of the vitellogenin mRNA. When this abundant cDNA was hybridized back to poly(A)⁺ RNA from which the cDNA was synthesized, 90% of the abundant cDNA hybridized at a $R_0t_{1/2}$ of $9 \times 10^{-3} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$. On the basis of the value of sequence complexity of 7000 for chicken vitellogenin mRNA of 7000 nucleotides (Gordon *et al.*, 1978), a pure preparation of poly(A)⁺ RNA comprising a single mRNA species would have reacted with a $R_0t_{1/2}$ of 5×10^{-3} , which indicates that only 55% of our poly(A)⁺ RNA actually represents the high-molecular-weight RNA.

Based on (a) a recovery of $11 \mu\text{g}$ of poly(A)⁺ RNA/g of fat-body, (b) the presence of about 1.7×10^8 nuclei/g of fat-body, (c) the complexity of poly(A)⁺ RNA calculated as 1.17×10^8 nucleotides/cell, and (d) 55% of the poly(A)⁺ RNA being vitellogenin mRNA, we have derived a value for the content of vitellogenin mRNA molecules as 9.1×10^3 copies per cell. This corresponds to a value of about 0.7% of the total RNA in fat-bodies of vitellogenic female locusts as vitellogenin mRNA. This high content of mRNA is consistent with the physiological demand for massive vitellogenin synthesis imposed on the vitellogenic fat-body during the short egg-laying cycles.

DNA-excess hybridizations

The preparation of cDNA to locust vitellogenin mRNA hybridized to total sheared locust genomic DNA at an equivalent C_0t value of $10^6 \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$ (Fig. 5a). By analogy with results for sea urchin (Graham *et al.*, 1974) and chicken (Eden & Hendrick, 1978), a reassociation rate constant for this reaction of $2 \times 10^{-3} \text{ mol} \cdot \text{litre}^{-1} \cdot \text{s}$ would indicate that gene(s) coding for locust vitellogenin is present as a 'single copy' component of the total DNA. In this context it is worth noting that an analysis of hybridization kinetics of this type does not allow one to distinguish between a truly single-copy gene and a small multi-gene family. Indeed, studies on *Xenopus* vitellogenin have revealed a small multi-gene vitellogenin family (Wahli *et al.*, 1979; Tata *et al.*, 1980). In cDNA–DNA hybridization, the fraction of cDNA hybridizing is dependent on the ratio of driver:tracer DNA. By using a 10^6 -fold excess of genomic DNA as driver, it was possible to achieve about 75% hybridization. The thermal-denaturation experiments (Fig. 5b) show a sharp 'melting' profile with a half-'melting' temperature (T_m) of 82°C , which is about 12°C above the hybridization temperature, indicating that the hybrids are well matched.

The cDNA prepared by the methods outlined in this paper has also been used for the determination of homology of locust DNA sequences coding for vitellogenin mRNA with similar sequences expressed in other organisms. In these studies, vitellogenin sequences of chicken, *Xenopus* and locust have been found to be a partially conserved set of sequences, on the basis of the cross-hybridization of cDNA against vitellogenin mRNA with genomic DNA from these three evolutionary very divergent species (T. C. James, S. W. Applebaum & J. R. Tata, unpublished work).

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Fig. 5. Hybridization of purified cDNA to locust vitellogenin mRNA with locust genomic DNA

(a) Kinetics of hybridization. Purified [³²P]cDNA (specific radioactivity 400 c.p.m./pg) was hybridized to a 10⁶-fold excess of sheared locust genomic DNA as described in the text. Equivalent C₀t values in 0.12 M-phosphate buffer were calculated as described by Britten *et al.* (1974). (b) 'Melting' profiles of locust genomic-DNA-vitellogenin cDNA hybrids, as determined by S₁-nuclease resistance. Purified [³²P]cDNA to locust vitellogenin was hybridized to a 10⁶-fold excess of total, sheared genomic DNA to an equivalent C₀t value of 10⁶ in 50 mM-Tris/HCl (pH 7.0)/0.3 M-NaCl/1 mM-EDTA/0.1% SDS. Thermal denaturation was performed as described in the text.

References

- Applebaum, S. W., Ebstein, R. P. & Wyatt, G. R. (1966) *J. Mol. Biol.* **21**, 29–41
- Bergink, E. W. & Wallace, R. A. (1974) *J. Biol. Chem.* **249**, 2897–2903
- Bishop, J. O., Miston, J. B., Rosbash, M. & Richardson, M. (1974) *Nature (London)* **250**, 199–204
- Britten, R. J., Graham, D. E. & Neufeld, B. R. (1974) *Methods Enzymol.* **29**, 363–405
- Chen, T. T. (1980) *Arch. Biochem. Biophys.* **201**, 266–276
- Chen, T. T., Strahlendorf, P. W. & Wyatt, G. R. (1978) *J. Biol. Chem.* **253**, 5325–5331
- Couple, P., Chen, T. T. & Wyatt, G. R. (1979) *J. Insect Physiol.* **25**, 327–337
- Deeley, R. G., Gordon, J. I., Burns, A. T. H., Mullinix, K. P., Binastein, M. & Goldberger, R. F. (1977) *J. Biol. Chem.* **252**, 8310–8319
- Eden, F. C. & Hendrick, J. P. (1978) *Biochemistry* **17**, 5838–5844
- Engelmann, F. & Friedel, T. (1974) *Life Sci.* **14**, 587–594
- Gellissen, G., Wajc, E., Cohen, E., Emmerich, H., Applebaum, S. W. & Flossdorf, T. (1976) *J. Comp. Physiol. B* **108**, 287–301
- Gordon, J. I., Burns, A. T. H., Christmann, J. L. & Deeley, R. G. (1978) *J. Biol. Chem.* **253**, 8629–8639
- Graham, D. E., Neufeld, B. N., Davidson, E. H. & Britten, R. J. (1974) *Cell* **1**, 127–137
- Gross-Bellard, M., Oudet, P. & Chambon, P. (1973) *Eur. J. Biochem.* **36**, 32–38
- Gruber, M., Bos, E. S. & AB, G. (1976) *Mol. Cell. Endocrinol.* **5**, 41–50
- Harry, P., Pines, M. & Applebaum, S. W. (1979) *Comp. Biochem. Physiol.* **63B**, 287–293
- James, T. C. & Tata, J. R. (1980) *Differentiation* **16**, 9–23
- Kelly, T. J. & Telfer, W. H. (1977) *Dev. Biol.* **61**, 58–69
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Laskey, R. A., Miller, A. D. & Morris, N. R. (1977) *Cell* **10**, 237–243
- McMaster, G. K. & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4835–4838
- Milcarek, C., Price, R. & Penman, S. (1974) *Cell* **3**, 1–10
- Mundall, E. C., Tobe, S. S. & Stay, B. (1979) *Nature (London)* **282**, 97–98
- Ohno, T., Cozens, P. J., Cato, A. C. B. & Jost, J. P. (1980) *Biochim. Biophys. Acta* **606**, 34–46
- Pelham, H. R. B. & Jackson, R. J. (1976) *Eur. J. Biochem.* **67**, 247–256
- Rhoads, R. E. (1975) *J. Biol. Chem.* **250**, 8088–8097
- Roskam, W. G. & Rougeon, F. (1979) *Nucleic Acids Res.* **7**, 305–320
- Shapiro, D. J. & Schimke, R. T. (1975) *J. Biol. Chem.* **250**, 1759–1764
- Shore, G. C. & Tata, J. R. (1977) *J. Cell Biol.* **72**, 726–743
- Smith, D. F., Searle, P. F. & Williams, J. G. (1979) *Nucleic Acids Res.* **6**, 487–506
- Tata, J. R. & Baker, B. (1975) *Exp. Cell Res.* **93**, 191–201
- Tata, J. R. & Smith, D. F. (1979) *Recent Prog. Horm. Res.* **35**, 47–95
- Tata, J. R., Baker, B. S. & Deeley, J. V. (1980) *J. Biol. Chem.* **255**, 6721–6726
- Telfer, W. H. (1954) *J. Gen. Physiol.* **37**, 539–558
- Vassart, G., Brocas, H., Nokin, P. & Dumont, J. E. (1973) *Biochim. Biophys. Acta* **324**, 575–580
- Wahli, W., Ryffel, G. U., Wyler, T., Jaggi, R. B., Weber, R. & Dawid, I. B. (1978) *Dev. Biol.* **67**, 371–383
- Wahli, W., Dawid, I. B., Wyler, T., Jaggi, R. B., Weber, R. & Ryffel, G. U. (1979) *Cell* **16**, 535–549
- Williams, J. G. & Penman, S. (1975) *Cell* **6**, 197–206